Synergistic activity of insulin combined with glucose on *Toxoplasma gondii* proliferation in Vero cells

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To the Editor: *Toxoplasma gondii* is a widespread apicomplexan parasite and can infect virtually all warm-blooded vertebrates.[1] Between 22% and 84% of the population in both developed and developing countries is chronically infected with *T. gondii*. Due to the ethical issues of using animal models for *in vivo* culture, cell culture systems have been used. Production of *T. gondii* tachyzoites *in vitro* is essential for *Toxoplasma* investigations. It is of great significance to explore the culture conditions of *T. gondii in vitro* for the in-depth study of the metabolism mechanisms of eukaryotes.

The study demonstrated that *T. gondii* tachyzoites can be cultured and maintained in various culture systems including the Vero cell line. Insulin is an acidic protein that regulates the metabolism of glucose, protein, and fat as well as the biological function of cells. Its action pathway is that insulin first binds to receptors on the cell membrane to initiate a series of protein phosphorylation reactions, consequently controlling glucose absorption and metabolism, and at the same time, glucose affects cell division and proliferation with its long-term effects.[2] According to a report, not only insulin but also effective fragments of insulin have the function of promoting glucose absorption and stimulating cell proliferation. However, whether insulin and glucose have synergistic activity promoting *T. gondii* replication is not clear. The purpose of this study is to improve the culture conditions to obtain more viable *T. gondii*.

Vero cells (American Type Culture Collection, ATCC) were cultured in Dulbecco modified Eagle medium (DMEM; Corning University Boulevard, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) in a humidified incubator at 37°C and 5% CO_2. After reaching confluence, the monolayer cells were rinsed with phosphate-buffered saline (PBS), resuspended in trypsin, quenched with host cell medium, and passed into fresh flasks.

Tachyzoites were inoculated intraperitoneally into BALB/c mice and harvested by PBS after 3 to 4 days of growth. These parasites were then cultured in human Vero cells maintained in DMEM medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS at 37°C and 5% CO_2. Vero cells were seeded in 24-well plates and cultured for 48 h. After the formation of confluent monolayers, cells were infected by 1×10^5 tachyzoites of *T. gondii*, RH strain (type I representative strain). The culture media were replaced by DMEM without fetal calf serum. One hour after infection, *T. gondii* that did not enter the cells was washed off with DMEM, and the media were replaced by 500 μL/well of DMEM containing various concentrations of glucose (1, 2.5, 4.5, 10, and 20 mg/mL) and/or insulin (10^{-3}, 10^{-2}, 10^{-1}, 1, and 10 μg/mL). The media were changed daily. The number of tachyzoites was calculated on the second, third, fourth, fifth, and sixth day when the cells were completely ruptured.

We explored the effect of various concentrations of glucose and/or insulin on *T. gondii* intracellular proliferation in Vero cells [Figure 1A]. The study demonstrated that glucose has a dose-dependent effect on *T. gondii* replication in Vero cells. Compared with the control group, 4.5 mg/mL glucose has a significant promoting effect on the growth of *T. gondii*; however, it has an inhibitory effect when the concentration of glucose is >10 mg/mL. We also found that, compared with the control group, a lower concentration of insulin can also enhance *T. gondii* growth rate. When the insulin concentration is 10^{-1} μg/mL, the promoting effect is the best. The growth rate of *T. gondii* decreased with the increase of insulin concentration in the culture medium. Especially, when the concentrations of insulin are >10 μg/mL, the replication of *Toxoplasma* was obviously inhibited [Figure 1B]. In addition, it revealed that the function of low concentrations of insulin such as

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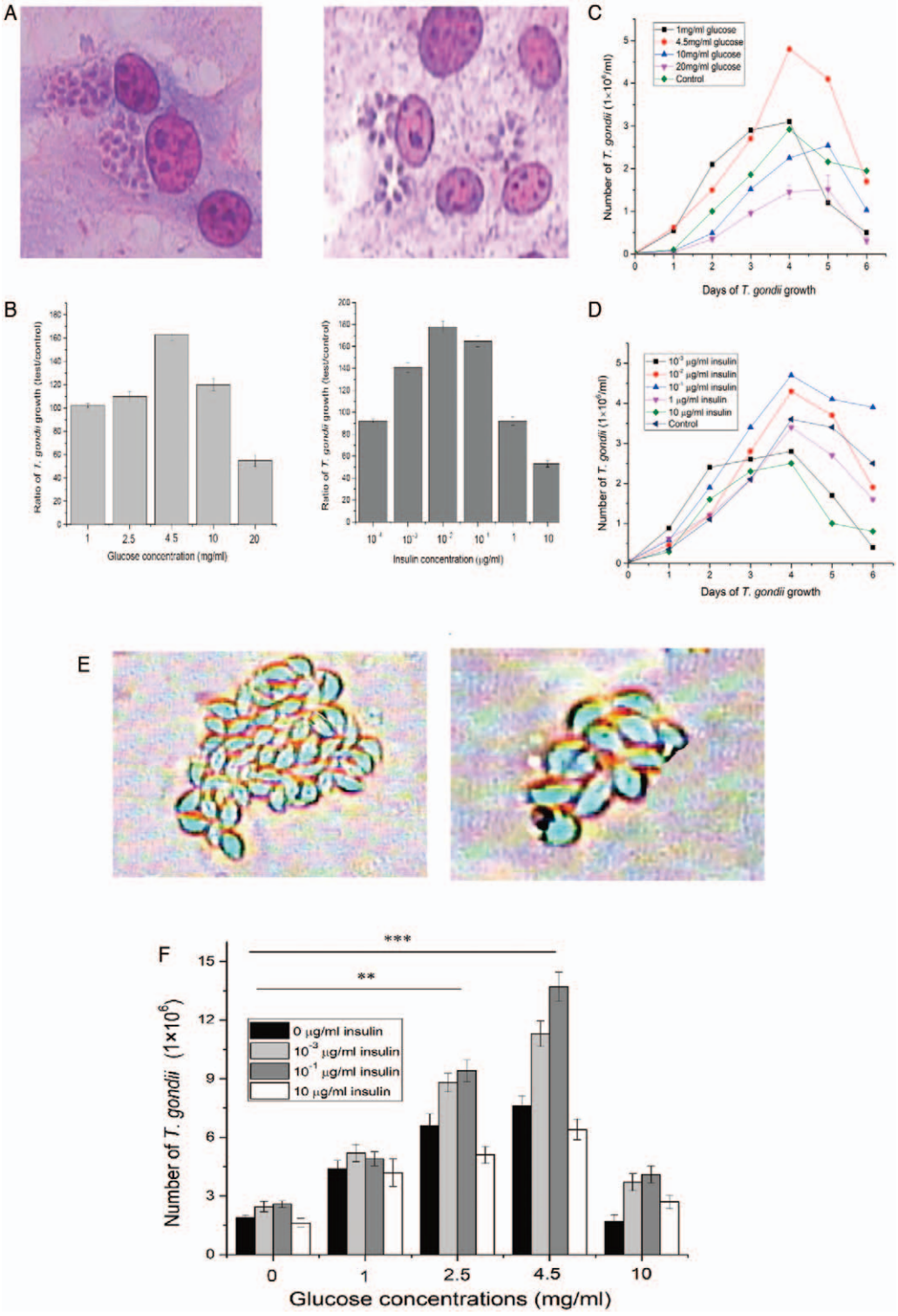

Figure 1: Mitogenic effect of insulin and/or glucose on *Toxoplasma gondii* replication in Vero cells. (A) Image of the tachyzoites in Vero cells cultured in the DMEM contained 4.5 mg/mL glucose combined with 10^{-1} μg/mL insulin. (B) Treatment with various concentrations of glucose or insulin. (C) Effect of different concentrations of glucose on *T. gondii* replication in Vero cells. (D) Effect of different concentrations of insulin on *T. gondii* replication in Vero cells. (E) Representative images of the free tachyzoites after Vero cell lysis in the media contained 4.5 mg/mL glucose and 10^{-1} μg/mL insulin. (F) Synergistic effect of various concentrations of glucose combined with various concentrations of insulin on *T. gondii* replication in Vero cells. $n = 4$. $^{**}P < 0.01$, $^{***}P < 0.001$. DMEM: Dulbecco modified Eagle medium.

10^{-4} μg/mL mainly focuses on the early stage of *T. gondii* replication.

By counting the number of *T. gondii* at different time, it was found that different concentrations of glucose from 1 to 4.5 mg/mL could significantly promote the growth of tachyzoites; however, 20 mg/mL of glucose has an obvious inhibitory effect compared with the control group [Figure 1C]. We found that after 24 h of treatment by different concentrations of insulin, there was no significant

difference in the amount of the tachyzoite. With the increase in culture time, insulin has a significant negative effect on the proliferation of *T. gondii*. Insulin in the range of 10^{-2} μg/mL to 1 μg/mL can promote the growth of *T. gondii* in cells, while it shows a significant inhibitory effect when the concentrations are >10 μg/mL [Figure 1D]. Tachyzoites reached the maximum concentration on the fourth day and thereafter began to decline rapidly.

Infected Vero cells were treated by various concentrations of glucose combined with various concentrations of insulin, and after 4 days of culture when the cells were completely ruptured, the number of tachyzoite in the media was counted. It can be seen in Figure 1E, when the mixture contains 10^{-1} μg/mL insulin and 4.5 mg/mL glucose, the proliferation of *T. gondii* is significantly increased compared with the group free of glucose and insulin [Figure 1E]. The combination of 10^{-1} μg/mL insulin and 2.5 mg/mL glucose has a similar effect on tachyzoite growth ($8.31 \pm 0.35 \times 10^6$/mL). Regardless of the glucose concentration, high concentrations of insulin are detrimental to the growth of *T. gondii*. It was found that when the concentration of insulin was 10^{-1} μg/mL, the tachyzoites growth state is the best. *T. gondii* increases 4.6 times in the group of 4.5 mg/mL glucose combined with 10^{-1} μg/mL insulin compared with the insulin and glucose-free group [Figure 1F].

Our results demonstrated that a combination of insulin and glucose could synergistically accelerate *T. gondii* growth and replication in Vero cells. The function of insulin in humans has many facets. Insulin controls or alters glucose, protein, and fat metabolism as well as other cellular functions. However, the effects of insulin on the entire organism are manifold and complex. Insulin causes, for instance, the translocation of glucose from intracellular vesicles to the cell membrane and, thus, increases the rate of glucose entry for a given concentration into the target tissues.

By binding to a specific receptor on the cell membrane, insulin initiates protein phosphorylation cascade, thereby controlling glucose uptake and metabolism.[3] Studies have shown that many tissues and organs express insulin receptors and initiate various intracellular actions. Insulin is a key component of the most fully defined media devised for mammalian cell culture.[4] Many microorganisms produce specific metabolic or mitotic responses to mammalian insulin; some of them have been shown to synthesize insulin-like molecules.[5] These phenomena have been revealed in several unicellular eukaryotes such as *Tetrahymena*, which was particularly widely studied. However, relatively little work has been focused on the growth of *T. gondii* in this respect.

In conclusion, insulin in combination with glucose plays an important role in stimulating *T. gondii* growth in Vero cells. However, little is known about signal transduction pathways in *T. gondii*, though recent genome and transcriptome analyses have given evidence that serine-threonine protein kinase (AKT) signaling pathway is essential for the development of the schistosome and may be involved in the regulation of their complex life cycle and the interactions with the hosts, the mechanisms of insulin stimulation and how *T. gondii* uses glucose as an energy source need to be further explored.

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Conflicts of interest

None.

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